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Review

Progranulin: A promising therapeutic target for rheumatoid arthritis

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ABSTRACT

Progranulin (PGRN) is an autocrine growth factor with multiple functions. This review provides updates about the interplays of PGRN with extracellular matrix proteins, proteolytic enzymes, inflammatory cytokines, and cell surface receptors in cartilage and arthritis, with a special focus on the interaction between PGRN and TNF receptors (TNFR) and its implications in inflammatory arthritis. The paper also highlights Atsttrin, an engineered protein composed of three PGRN fragments that prevents inflammation in several inflammatory arthritis models. Identification of PGRN as a ligand of TNFR and an antagonist of TNFα signaling, together with the discovery of Atsttrin, not only betters our understanding of the pathogenesis of arthritis, but also provides new therapeutic interventions for various TNFα-mediated pathologies and conditions, including rheumatoid arthritis.

1. Introduction

Progranulin (PGRN), also known as granulin epithelin precursor (GEP), PC-cell-derived growth factor (PCDGF), proepithelin, and acrogranin, is a 593-amino-acid autocrine growth factor. PGRN contains seven-and-a-half repeats of a cysteine-rich motif ($CX_{5-6}CX_5CCX_8CCX_6CCXDX_2HCCPX_4CX_{5-6}C$) in the order P–G–F–B–A–C–D–E, where A–G are full repeats and P is the half-motif [1]. PGRN is abundantly expressed in rapidly cycling epithelial cells, in cells of the immune system, in neurons [2], and in chondrocytes [3]. High levels of PGRN expression are also found in varieties of human cancers and contribute to tumorigenesis in breast cancer, ovarian carcinoma, and multiple myeloma [2,4]. PGRN is known to play a critical role in a variety of physiologic and disease processes, including early embryogenesis [5], wound healing [6], inflammation [7,8], and host defense [9]. PGRN also functions as a neurotrophic factor [10,11] and mutations in the *PGRN* gene resulting in partial loss of the PGRN protein cause frontotemporal dementia [12–14]. PGRN was also isolated as an important regulator of cartilage development and degradation [3,15–17]. For the detailed introduction concerning the structure and function of PGRN, please see the review paper written by Drs. Bateman and Bennett [2]. In this paper, I will briefly survey the association of PGRN with its binding partners in arthritis, with a special focus on the interaction between PGRN and TNFR as well as the discovery of PGRN-derived Atsttrin that effectively prevents the onset and progression of inflammatory arthritis [18–20].

2. PGRN associates with extracellular matrix proteins

The extracellular matrix of cartilage consists of several types of collagens, proteoglycans, and other non-collagenous macromolecules, all of which interact to form a highly specialized connective tissue [21]. Arthritis is a disease process characterized by the proteolytic degradation of extracellular matrix components with subsequent loss of articular cartilage and bone. Cartilage oligomeric matrix protein (COMP), a prominent non-collagenous component of cartilage, accounting for approximately 1% of the tissue's wet weight, has also been localized in tendon, bone (osteoblasts only), and synovium [22–25]. COMP is a 524 kDa pentameric, disulfide-bonded, multidomain glycoprotein composed of approximately equal subunits (~110 kDa each) [22,26]. Fragments of COMP have been detected in diseased cartilage, synovial fluid, and serum of patients with knee injuries, posttraumatic, primary osteoarthritis (OA), and rheumatoid arthritis (RA) [27–29]. Monitoring of COMP levels in either joint fluid or serum can be used to assess the presence and progression of arthritis [30–35]. Mutations in the human COMP gene have been linked to the development of pseudoachondroplasia and multiple epiphyseal dysplasia, autosomal-dominant forms of short-limb dwarfism characterized by short stature,

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normal facies, epiphyseal abnormalities, and early-onset OA [36–42]. PGRN was identified as a COMP-binding growth factor in an effort to define the biological functions of COMP [16]. PGRN directly binds to COMP both in vitro and in vivo. PGRN selectively interacts with the EGF repeat domain of COMP but not with the COMP's other three functional domains. The Granulin A repeat unit of PGRN is required and sufficient for association with COMP. Overexpression of PGRN stimulates the proliferation of chondrocytes and this stimulation is enhanced by COMP. In addition, COMP appears to be required for PGRN-mediated chondrocyte proliferation, since chondrocyte proliferation induced by PGRN is dramatically inhibited by an anti-COMP antibody.

Extracellular matrix protein 1 (ECM1), a COMP-associated matrix protein [43], was also found to interact with PGRN. COMP and ECM1, however, exert an opposite effect on PGRN cell surface localization: COMP enhances, whereas ECM1 inhibits, cell surface appearance of PGRN (Kong and Liu, unpublished data). Perlecan, a heparan sulfate proteoglycan important for chondrocyte differentiation and function [44,45], was known to bind to ECM1 [46]. Interestingly, PGRN was also reported to associate with perlecan, and the PGRN–perlecan interaction was suggested to modulate tumor growth [47]. Whether this interaction is also important for the PGRN activity in cartilage and arthritis remains to be delineated.

3. PGRN associates with proteolytic enzymes

During inflammation, neutrophils and macrophages release serine proteases that digest PGRN into individual 6 kDa granulin units, which are actually pro-inflammatory and can neutralize the anti-inflammatory effects of intact PGRN [7,8]. Both neutrophil elastase (NE) and proteinase 3 (PR3) are known to digest PGRN at its linker regions, resulting in the liberation of individual granulin units [7,8], and are involved in the PGRN conversion during neutrophil activation in vitro and immune complex-mediated inflammation in vivo [7,48]. PGRN's anti-inflammatory actions are protected by its binding proteins, which include the secretory leukocyte protease inhibitor [8] and apolipoprotein A1 [49], both of which bind to PGRN and protect it against proteolytic degradation.

In addition to serine proteases, several metalloproteinases from both ADAMTS and MMP families were also found to associate with and degrade PGRN [3,15,50–52]. PGRN binds directly to ADAMTS-7 and ADAMTS-12 in vitro and in chondrocytes, and the four C-terminal TSP motifs of ADAMTS-7/-12 and each granulin unit of PGRN mediate their interactions. Additionally, PGRN co-localizes with ADAMTS-7 and ADAMTS-12 on the cell surface of chondrocytes [15,50]. ADAMTS-7 acts as a new PGRN-convertase and neutralizes PGRN-stimulated endochondral bone formation [50]. More significantly, PGRN inhibits COMP degradation by ADAMTS-7/-12 through the following two mechanisms: (a) competitive inhibition through direct protein-protein interactions with ADAMTS-7/-12 and COMP; and (b) inhibition of TNFα-induced ADAMTS-7/-12 expression [15,17]. Furthermore, PGRN levels are significantly elevated in patients with either osteroarthritis or rheumatoid arthritis [15]. These observations demonstrate a novel protein–protein interaction network among PGRN growth factor, ADAMTS-7 and ADAMTS-12 metalloproteinases, and COMP extracellular matrix protein. Furthermore, PGRN functions as a specific inhibitor of ADAMTS-7/-12-mediated COMP degradation and may play a significant role in preventing the destruction of joint cartilage in arthritis.

In addition, PGRN was also isolated as a novel substrate of the membrane type 1 matrix metalloproteinase (MMP-14) in a quantitative proteomic evaluation of the inhibitor of MMP-14 with isotope-coded affinity tag labeling and tandem mass spectrometry [52].

4. PGRN associates with cell surface receptors

4.1. PGRN directly binds to TNFR and antagonizes TNFα actions

Although PGRN plays crucial roles in multiple physiological and pathological conditions, efforts to exploit the actions of PGRN and understand the mechanisms involved have been hampered by our inability to identify its binding receptor(s) [2]. To address this issue, we performed a global genetic screen that led to the identification of TNFR2 as the PGRN-associated receptor [18]. PGRN exhibited higher affinity for TNF receptors, especially TNFR2 when compared with TNFα. In contrast to TNFα, which demonstrated higher affinity for TNFR1 than TNFR2, PGRN exhibited comparable binding affinity for TNFR1 and TNFR2 [18]. PGRN acts as a physiological antagonist of TNFα signaling and disturbs the binding of TNFα and TNFR [18]. Previous reports have demonstrated that PGRN potently inhibits TNF-mediated neutrophil activation [8] and cartilage degradation [3]. In addition, the deletion of PGRN led to a significant increase in hydrogen peroxide in neutrophils and nitric oxide in bone marrow derived macrophages, and a marked increase in TNFα-induced COMP degradation in cartilage explants [18].

4.2. PGRN-deficient mice are susceptible to collagen-induced arthritis, and administration of PGRN abolishes the severe inflammatory arthritis seen in collagen-challenged PGRN-deficient mice

To examine the role of endogenous PGRN during inflammation in vivo, we investigated the clinical and histopathological features of PGRN$^{-/-}$ mice and their control littermates in a mouse model of collagen-induced arthritis (CIA) [53,54]. PGRN$^{-/-}$ mice immunized with collagen II developed higher severity inflammatory arthritis and increased bone and joint destruction as compared with their control littermates. We also observed a significant increase in the arthritis severity score and a 100% incidence of arthritis in PGRN$^{-/-}$ mice, which was dramatically higher when compared with the 40% arthritis incidence of wild-type control CIA C57B6 mice. Importantly, administration of recombinant PGRN completely blocked disease progression in PGRN-deficient CIA mice. Collectively, these data suggest that the loss of PGRN expression in vivo results in hyper-susceptibility to collagen induced arthritis, which can be entirely reversed by the administration of recombinant PGRN [18].

4.3. Deletion of the PGRN gene exacerbates, whereas recombinant PGRN prevents, the spontaneous development of inflammatory arthritis in TNF transgenic mice

To determine whether the anti-inflammatory actions of PGRN occur through the suppression of TNFα signaling in vivo, we deleted the *PGRN* gene in TNF transgenic mice (TNF-Tg). TNF-Tg mice are known to develop an inflammatory arthritis phenotype spontaneously [55,56]. The deletion of PGRN markedly hastened the onset of arthritis. 12-week-old TNF-Tg/PGRN$^{-/-}$ and TNF-Tg/PGRN$^{+/-}$ mice developed severe swelling and joint deformation, which contributed to a significant loss of mobility. In contrast, TNF-Tg mice developed only mild signs of inflammation. TNF-Tg/PGRN$^{-/-}$ and TNF-Tg/PGRN$^{+/-}$ mice demonstrated significantly increased synovitis, pannus formation, destruction of the ankle joints, and loss of cartilage matrix. Treatment of TNF-Tg mice with recombinant PGRN resulted in the elimination of any visual signs of arthritis and a dramatically reduced arthritis severity score. Interestingly, at seven days following the cessation of PGRN treatment, signs of arthritis began to develop. Taken together, these data suggest that PGRN may exert its anti-inflammatory effects through, at least in part, the inhibition of TNF/TNFR signaling in vivo.

4.4. PGRN binds to sortilin in neuron

Recently, Hu and colleagues identified sortilin as a neuronal receptor for PGRN that facilitates its endocytosis and regulates PGRN levels in vitro and in vivo [57], suggesting that sortilin-mediated PGRN endocytosis may play a role in the pathophysiology of the neurodegenerative disease frontotemporal lobar degeneration [57,58]. Sortilin, highly enriched in the vertebrate central nervous system [59], is also known to interact with the neural growth factors, including nerve growth factor (NGF) [60] and brain-derived neurotrophic factor (BDNF) [61]. In addition, sortilin also binds to other polypeptides such as neurotensin, sphingolipid activator protein and lipoprotein lipase and has been suggested to be involved in proper intracellular trafficking of these polypeptides [62–66].

5. Discovery of PGRN-derived Atsttrin

Although PGRN has anti-inflammatory activity [7,8,18], it is unlikely that PGRN can be directly employed for treating inflammatory disorders due to its multiple functions, especially its oncogenic activity [2]. Thus, we put a lot of effort into constructing a molecule that remains TNFR binding but loses PGRN's oncogenic action. We started with the identification of the domains of PGRN required for its interaction with TNF receptors. A series of PGRN mutants were constructed and analyzed for their interactions with TNFR2. No single granulin unit (A–G) or linker region was able to bind to TNFR2, suggesting that the binding domain of PGRN may span granulin unit and linker. To examine this hypothesis, we first expressed each granulin with its immediately adjacent downstream linker and found that granulin F plus linker P3 exhibited a weak interaction with TNFR2. Next, we linked each granulin to its immediately adjacent upstream linker; P4-granulin A and P5-granulin C both demonstrated weak binding affinity to TNFR2. Finally, we linked all three fragments identified above to generate an engineered mutant (referred to as FAC) [18]. These data are in accordance with the findings that granulin F, A, and C are the granulin domains most capable of independent folding and that these granulin domains have N and C terminal subdomains that are structurally independent by NMR [67]. Interestingly, FAC exhibited an even stronger binding affinity to TNFR2 than PGRN [18]. To identify the minimal engineered mutant protein with retained binding affinity, we generated the mutant 2/3 (FAC), which is identical to FAC except that only 2/3 of each granulin unit was included. We observed that 2/3 (FAC) binds to TNFR2 with a lower affinity than FAC. A further reduction from 2/3 to 1/2 of each granulin unit did not alter the binding affinity; however, a reduction to 1/4 completely abolished the interaction with TNFR2. Taken together, these results suggest that a mutant composed of half units of granulins A, C, and F plus linkers P3–P5 appears to be the "minimal" engineered molecule that retains affinity to TNFR2. This molecule was referred to as Atsttrin (Antagonist of TNF/TNFR Signaling via Targeting to TNF Receptors) [18].

When compared with TNFα, recombinant Atsttrin exhibited higher binding affinity for TNFR2, but lower affinity for TNFR1. Similar to PGRN, Atsttrin inhibited the interaction between TNF and TNFR, and in turn, the downstream events of TNF/TNFR signaling, such as TNFα-induced proinflammatory cell activation, cytotoxicity, and osteoclastogenesis. In addition, Atsttrin exhibited potent anti-inflammatory responses in vivo. Administration of either PGRN or Atsttrin resulted in reduced disease severity in collagen antibody induced arthritis (CAIA) model, and both agents significantly delayed the progression of arthritis. Furthermore, Atsttrin was more effective than PGRN in delaying the onset of inflammation. In the CIA model, DBA/1 mice treated with PGRN or Atsttrin demonstrated markedly reduced pathology, with Atsttrin-treated mice bearing marked similarity to normal mice. In addition, both PGRN- and Atsttrin-treated mice were found to have decreased circulating levels of fragmentary COMP, a marker for cartilage breakdown. We also confirmed the therapeutic efficacy of Atsttrin in TNF transgenic mouse model. Consistent with the results observed in the CAIA and CIA models, the administration of Atsttrin markedly suppressed arthritis progression, and notably eliminated signs of inflammation. In addition, signs of inflammation returned following the cessation of Atsttrin treatment.

6. Comparison of Atsttrin with current TNF inhibitors

6.1. Unique mechanism of action

Since tissue destruction in RA is caused by inflammatory mediators, the currently approved biological therapies for RA treatment primarily target cytokines such as TNFα. Although treatment with these agents is highly effective in ameliorating disease and improving quality of life in some patients with moderate-to-severe disease, current TNFα inhibitors fail to provide effective treatment for up to 50% of RA patients [68]. Atsttrin demonstrates features that suggest it may compare favorably to these established agents. For example, all currently marketed anti-TNF therapies bind to the TNFα ligand, while, in contrast, Atsttrin binds to TNFR and not to TNFα itself. Due to this alternate mechanism of action, Atsttrin may be effective for the patients who fail to respond to current TNFα blockers [68]. The potential advantages of an 'anti-TNFR' approach (in contrast to an 'anti-TNF' approach) to the treatment of human chronic inflammatory and autoimmune conditions have been previously reviewed [69]. In addition, drugs such as Anakinra and Actemra, which target the IL-1 receptor and IL-6 receptor, have demonstrated that the selective targeting of cytokine receptors can deliver a highly effective clinical outcome [70].

6.2. Activation of TNFR2 protective pathway

Growing evidences indicate that TNFR2 signaling has a protective role in inflammatory arthritis and joint erosion [71,72]. It appears that PGRN is the optimal ligand for TNFR2, in terms of both biochemical (PGRN exhibits approximately 600-fold higher binding affinity to TNFR2 than TNFα) and functional evidences (PGRN and TNFR2 mediate beneficial and protective roles in the inflammatory processes [7,8,71,72]). Treg cells that only express high levels of TNFR2 but do not express TNFR1 [73–75] were known to play a critical role in the prevention of autoimmunity and other pathological immune responses [76,77]. PGRN significantly protects Treg from a negative regulation by TNFα in a dose-dependent manner [18,75]. In addition, PGRN selectively promotes the differentiation of Treg from Naïve T cells [18]. Furthermore, TNFR2$^{-/-}$ CIA mice are less sensitive to Atsttrin treatment although Atsttrin exhibits therapeutic effects in either of TNFR-null CIA mice [18]. Collectively, PGRN and probably its derived Atsttrin, exert their protective effects in the pathogenesis of mouse models of inflammatory arthritis through multiple mechanisms: (1) acting as antagonists of TNF-mediated inflammatory response; (2) regulating the functions of Treg in a TNFR2-dependent manner; and (3) stimulating Treg differentiation. Thus, PGRN is not only a novel antagonist of TNFα, but an important mediator of the immune system as well.

6.3. Tumor suppression activity

Study indicates that administration of current TNF blockers, such as Humira (adalimumab) and Remicade (infliximab), increases the cancer risk [78–81], but the underlying molecular

mechanism remains unclear. Identification of PGRN oncogenic growth factor [2,82–85] as a novel ligand of TNFR may help us to understand this puzzling phenomenon. As illustrated in Fig. 1, PGRN and TNF, two ligands of TNFR, reach balance under physiological conditions. If the balance is disturbed, for instance higher TNF activity, it will result in inflammation and autoimmune diseases, including rheumatoid arthritis. In contrast, if PGRN activity is too high, this may cause excessive cell growth and tumorgenesis (Fig. 1A). Current TNF inhibitors all bind to TNF, and inhibit TNF-mediated inflammation. Once the TNF ligand is blocked, more TNFR will become available to a second ligand, i.e., PGRN. In other words, the PGRN activity may be enhanced, which may result in increased cancer incidence (Fig. 1B). Atsttrin, which directly binds to TNFR rather than TNF ligand, does not increase cancer incidence. In contrast it actually acts as a tumor suppressor and has potential for treating cancers in which PGRN is highly active, such as liver and breast cancers (Fig. 1C). Indeed, Atsttrin has been shown to inhibit PGRN-stimulated cell proliferation of several cancer cell lines tested (Tang and Liu, unpublished data).

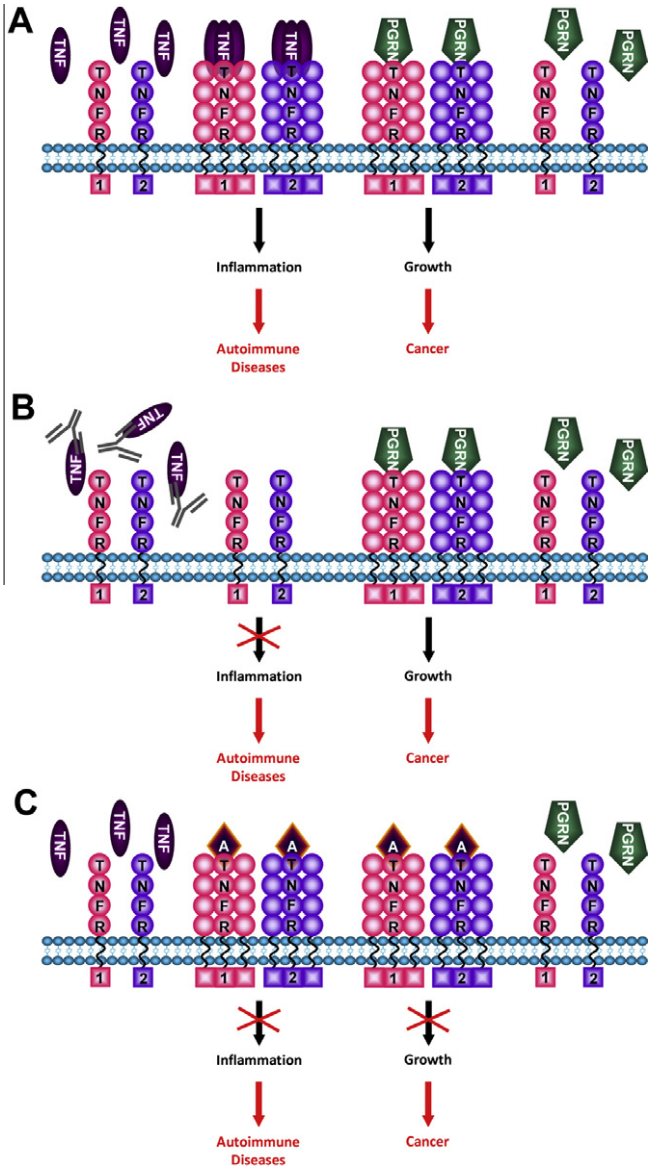

Fig. 1. Proposed model for comparing TNFα blockers and Atsttrin in mediating inflammation and cancer. (A) PGRN and TNFα, two ligands of TNFR that links inflammation and cancer, bind to TNFR. (B) Current TNFα inhibitors bind to TNFα and inhibit TNF-mediated inflammation. (C) Atsttrin, which directly binds to TNFR rather than TNFα, inhibits both inflammation and cancerogenesis.

6.4. Stability

The pharmacokinetic profile assay of Atsttrin in mice indicated that Atsttrin was well absorbed following intraperitoneal administration and demonstrated high stability with a half-life of about 120 h [18]. In accordance with its long half-life, a single dose of Atsttrin (10 mg/kg) could effectively delay the onset of inflammation for approximately three weeks [18]. PGRN-converting proteases, such as elastase and proteinase-3, are known to digest PGRN at its linker regions, resulting in the liberation of individual granulin units [7,8]. Interestingly, the presence of intact granulin units is required for PGRN to bind ADAMTS-7 and ADAMTS-12, two metalloproteinases known to associate with and digest PGRN [15,50]. Thus, the high stability of Atsttrin may be at least partially explained by its lack of intact granulins, which allows it to escape the proteolytic activity of degradative enzymes.

6.5. Efficacy

Atsttrin exhibits highly potent anti-inflammatory activity, which surpasses PGRN itself, in vivo [18]. This occurred despite the observation that PGRN binds to TNFR with a higher affinity than Atsttrin, a finding that may be explained by the fact that Atsttrin contains only partial granulin units and would not be expected to release any intact pro-inflammatory granulin units upon exposure to PGRN-converting enzymes such as elastase [8], proteinase-3 [7], and ADAMTS-7 [50]. In addition, Atsttrin exhibited a significantly longer half-life (∼120 h) when compared to PGRN (∼40 h). In addition, Atsttrin is also more efficacious than the current anti-TNFα therapy, including etanercept (Enbrel) and adalimumab (Humira), in several preclinical inflammatory arthritis models tested (Tang, et al., unpublished data) [18]. Whether the efficacy of Atsttrin observed in animal models can be translated into human patients remains to be determined.

6.6. Preclinical safety

Our results demonstrate that both PGRN and Atsttrin can prevent and ameliorate inflammatory arthritis in preclinical animal models without observable adverse effects [18]. Although this may present a case for advancing both agents to clinically based human study, we found several factors that may provide Atsttrin with an advantage over PGRN in the potential treatment of inflammatory disease. For instance, PGRN is known to be an oncogenic growth factor, and may contribute to tumorgenesis [2,86]. As described above, not only did Atsttrin not possess oncogenic activity, but it may even function as a tumor-suppressor (Tang and Liu, unpublished data). Additionally, although PGRN exhibited a higher binding affinity to both TNFR1 and TNFR2 when compared with TNFα, Atsttrin demonstrated ∼10-fold higher binding affinity for TNFR2, with a ∼18-fold lower affinity for TNFR1 when compared with TNFα [18]. We also observed that while PGRN could interact with several other members of the TNFR subfamily (although with lower affinity than TNFR), Atsttrin selectively interacted with TNFR1 and TNFR2, with a higher binding affinity to TNFR2 [18]. This would suggest that the administration of Atsttrin would potentially result in fewer adverse events than that of PGRN. Indeed, Atsttrin did not exhibit any cytotoxic effects, even at exceedingly high dosages in rhabdomyosarcoma A673/6 cells. Furthermore, DBA1/J mice were injected daily with a high dose of Atsttrin, and the heart, lung, stomach, spleen, pancreas, small intestine, and colon were collected with no observed defects (Tang

and Liu, unpublished data). No overt toxicity or lethality related to Atsttrin administration was observed throughout our study.

7. Conclusion and perspective

The understanding of the precise etiology, pathogenesis and progression of arthritic diseases remains beyond our reach. Using a functional genomic approach combined with biochemistry, cellular biology, and molecular biology techniques, PGRN growth factor has been isolated and shown to physically associate with extracellular matrix proteins (COMP [16], ECM1 [43], Perlecan [47]), protelytic enzymes (elastase [8] and proteinase 3 [7], ADAMTS-7 [50], ADAMTS-12 [15]), and TNF receptors (TNFR1 and TNFR2 [18]). Thus, PGRN and its binding partners constitute interplay networks and act in concert in mediating the pathogenesis of arthritis [17]. In addition, we have discovered and produced an engineered PGRN-derived protein, Atsttrin, which effectively prevents the onset and progression of inflammatory arthritis in several preclinical animal models [18]. With the consideration that TNFR signaling is involved in a plethora of disease processes, manipulation of new antagonists of the TNF/TNFR pathway may lead to innovative therapeutics for various pathologies and conditions, such as rheumatoid arthritis.

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